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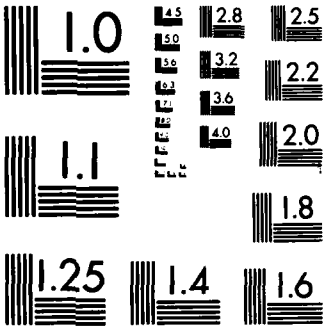

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The research objectives of the project are to develop genetic and molecular techniques that will permit the detection, isolation, and cloning of genes that are regulated during acetate catabolism. These studies should provide a firm basis for understanding the regulation of acetate utilization in the methanogen, Methanosarcina acitivorans. We have concentrated on three areas of study in the first year of the contract. They are; 1) development of cell plating methods for the methanosarcina, 2) screening and isolation of plasmids from the acetogenic methanogens, and 3) construction of gene libraries for M. acetivorans. It is anticipated that techniques developed in these studies will facilitate genetic study of other methanogenic organisms.

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Title: Gene Regulation of Methanogenesis from Acetate in the Acetotrophic Methane Producing Archaebacteria.

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Los Angeles, CA 90024

Period of Performance: 4/1/86 - 6/31/87

Table of Contents

Title: Gene Regulation of Methanogenesis from Acetate in the Acetotrophic Methane Producing Archaebacteria.

1. Project Goals

The goal of this project is to characterize the effect of methylotrophic substrates on the regulation of genes involved in acetate metabolism in M. acetivorans. We wish to develop genetic and molecular techniques for use in the Methanosarcina sp. that will allow us to detect, isolate and clone genes that are regulated during acetate catabolism. A search for plasmids in the methanosarcina will be performed. We will then attempt to develop a gene transfer system in M. acetivorans that would provide a powerful tool for the manipulation of genes necessary for acetate metabolism. Cell plating procedures will be developed for the M. acetivorans strain. Methods will be devised for isolation of large molecular weight genomic DNA and total genomic DNA libraries will be prepared. These studies should provide a firm foundation for addressing questions concerning the regulation of acetate utilization by methanogens. It is anticipated that techniques developed in these studies will facilitate genetic study of other methanogenic organisms.

2. Accomplishments

A. Cell plating of M. acetivorans.

Initial cell plating experiments have been conducted with the acetotroph M. acetivorans C2A. This species is one of the few Methanosarcina that can grow as single cells in contrast to the multicellular aggregates that are typical of the other Methanosarcina species. Using the general approach devised by Bertani and Baresi for the hydrogen utilizing organism Methanococcus voltae, we have succeeded in growing colonies of M. acetivorans on petri dishes using the following protocol. M. acetivorans is first cultured in liquid marine trimethylamine medium using conventional Hungate technique. At appropriate cell density, cultures are transferred into a Coy type anaerobic hood, cells are diluted into fresh marine trimethylamine medium and then plated at varying density on marine medium supplemented with purified agar (2 % w/v). Roll tubes are prepared at similar dilutions to compare the efficiency of cell survival. Cell counts are determined using a Petroff chamber to determine total cell number. We have modified Tobal anaerobe jars so that the sulfide concentration in the jars can be controlled. We have optimized plating conditions by varying the parameters of medium composition and incubation (i.e. method of sulfide supplementation, method of soft agar overlay, type of chamber used, time and temperature of incubation). We have determined the sulfide, carbon dioxide and agar concentrations required for maximum growth efficiency. We have also determined that an 0.5 % agar overlay method results in greater growth efficiency than the spreading method. The plating efficiency is routinely about 50 % to 95 % when compared to total cell counts. This corresponds to 100 % efficiency when compared to viable counts in roll tubes and dilutions in liquid medium. We have also devised replica plating techniques with M. acetivorans.

We have tested the effect of antibiotics and heavy metals on growth of M. acetivorans. Generally the methanogens are not sensitive to the antibiotics whose resistance genes are commonly used as genetic markers in the eubacteria and eukaryotes. However recent reports by other investigators indicate that the antibiotics thiostrepton, neomycin and bacitracin do effect growth of Methanococcus voltae. Neomycin had no effect on growth of M. acetivorans at concentrations up to 1000 μg/ml. However bacitracin and thiostrepton inhibited growth at concentrations as low as 100 μg/ml. These results are encouraging since genes that confer resistance to these antibiotics are available and could in principle be cloned into plasmid pC2A (see below) in an attempt to produce a hybrid shuttle vectror.

B. Isolation of plasmids in M. acetivorans.

Screening and isolation of plasmids from acetogenes. A potentially important tool for the study of gene regulation in methanogens is the use of recombinant DNA engineered vectors. Although plasmids and a phage have been isolated from the hydrogen-utilizing methanogenic species M. voltae and Methanobacterium thermoautotrophicum, none have been described from any of the acetate utilizing species. Additionally, neither transformation nor transduction methods have been developed in any of these methanogens. In order to construct gene mobilization vectors in the acetotrophic methanogenic bacteria, we have screened for the presence of plasmids.

Twelve methanogens that use acetate and methylated amines were screened. They are: M. acetivorans strain C2A, marine isolates C2B, C2C, C2D, C2J, C2E, M. thermophila TM1, Methanosarcina barkeri MS, Methanosarcina mazei S6, Methanococcoidies methylutens TMA10, Methanolobus tindarius T3, and Methanococcus halophilus Z7982. Three strains were found to contain plasmid. One of the plasmid-bearing strains, M. acetivorans C2A, is the strain originally proposed for study in this grant and is the one being used for cell plating experiments. A procedure has been developed for large scale purification of this plasmid (designated pC2A). The size of the plasmid has been determined to be approximately 5100 base pairs based on electron microscopy. The plasmid has been tested for digestion with over 20 restriction enzymes to determine the DNA modification properties of the strain. A restriction map has been generated for pC2A. (Figure 1; manuscript in preparation).

Resistances to various antibiotics and heavy metals are often mediated by genes carried by plasmids. In an effort to determine the function of plasmid pC2A, M. acetivorans and non-plasmid-bearing strains were tested for sensitivities to antibiotics and metals previously reported to have an effect on other methanogens. However, no differences in resistance by these materials were observed for the plasmid containing vs plasmid free strains.

C. Gene Cloning

1) Isolation of large molecular weight chromosomal DNA from M. acetivorans.

The preparation of large molecular weight DNA from the Methanosarcina has been a major technical barrier to performing gene cloning experiments in these organisms because the harsh mechanical methods needed to rupture the cells also shear the nucleic acids. We have solved this isolation problem. Large molecular weight chromosomal DNA has now been successfully purified from 100 ml cultures of M. acetivorans and marine strains C2B, C2C, C2D, C2E, and C2J,

M. tindarius T3, M. methylutens TMA 10 and M. halophilus Z7982. Cultures were grown in marine trimethylamine medium, harvested by centrifugation, and lysed in buffered SDS. The DNA was then purified by a modification of the Marmur procedure. We have also succeeded in obtaining DNA from the heteropolysaccharide wall bearing Methanosarcina including M. thermophila TM-1, M. barkeri MS and M. mazei S6. We are currently refining our DNA preparation techniques and will present our findings in the next report. Our extensive chromosomal DNA collection represents a valuable source of material for the experiments proposed in this project. Analysis of M. acetivorans DNA preparations by agarose gel electrophoresis reveal that it is of sufficient quality and concentration for subsequent gene cloning experiments. Very little DNA shearing occured during the cell lysis and DNA purification steps. The DNA has been screened for digestibility by a number of restriction enzymes that will be used for genomic library construction experiments. The ability to purify high molecular weight DNA from M. thermophila will enable us to construct and probe genomic libraries of this organism for obtaining desired genes.

2) Construction of gene libraries for M. acetivorans.

Creation of gene libraries. We have proceeded with the generation of a gene library of M. acetivorans DNA. The phage vector, λgt11 was selected for the initial library construction because it can be used for gene screening by either oligonucleotide or antibody probe approaches. The λgt11 library of M. acetivorans DNA has the following properties: titer of 5×10^9 phage per ml; 70 % insert frequency; average insert size of 3 kilobases. Assuming that the M. acetivorans genome is 1000 to 1500 kilo basepairs, every gene (assuming an average size of 1 kb) in the chromosome should be represented on the library at least once and probably over ten times. We will proceed with gene cloning experiments when the amino acid sequencing studies are sufficiently advanced to allow the design and synthesis of oligonucleotide probes.

3) Amino acid sequencing of CO dehydrogenase.

We have obtained purified CO dehydrogenase from the methanogen, Methanosarcina thermophila strain TM-1 from J. G. Ferry of VPI in Blacksburg, VA. M. thermophila is closely related to M. acetivorans C2A phylogenetically and can use acetate as a substrate for methane generation. It is our hope that the genes for the CO dehydrogenase from strain TM-1 will be sufficiently related to the genes from the C2A strain such that they can be cloned using homologous gene probes. We are currently testing this possibility since it will save us considerable time in the cloning aspect of the experiments.

We are performing protein sequencing experiments on the CO dehydrogenase protein to determine the amino acid sequence of the subunits. The enzyme is estimated to be greater than 98 % pure and contains five subunits based on SDS polyacrilamide gel electrophoresis. Two of these subunits, 89 kd and 19 kd appear to be induced by growth on acetate based on research in the Ferry laboratory. We have purified the largest and smallest of the five subunits by SDS polyacrylamide gel electrophoresis. The protein was recovered by electroelution from the gel slice and was submitted for N-terminal sequence analysis. We have obtained a partial amino acid sequence for the N-terminal 18 residues of the 89 kd subunit and the 38 N-terminal amino acids for the 19k subunit. The goal of these experiments is to design and synthesize

oligonucleotides of about 18 bases in length that will be used as specific gene probes for the CO dehydrogenase genes in the M. acetivorans genomic library.

3. Conclusions and plans

We have refined the techniques for obtaining efficient growth of M. acetivorans on plates and we are currently testing various procedures for improving efficiency. We will then generate UV killing curves and begin to screen for mutants. Although plasmid pC2A remains cryptic at this time it may still be useful for production of a hybrid shuttle vector by inserting genes for antibiotic resistance or amino acid synthesis.

We also plan to continue sequence work on the 19K subunit of CODH. Experiments are already underway to determine codon usage in M. thermophila and M. acetivorans. This data will enable us to better decide which oligonucleotides to synthesize and use for probing our libraries for CODH genes. Once cloned, it will be possible to then examine the regulation of these genes in response to environmental conditions.

4. Personnel

Dr. Kevin Sowers, a Postdoctoral Fellow, is supported in part by this grant. He is responsible for the plasmid isolation studies, the chromosomal DNA isolations and gene library work, in addition to the gene cloning studies.

Ms. Jane Boone is a Staff Research Associate and has performed the cell plating experiments with Methanosarcina acetivorans.

In addition, two graduate students have received training in the methods of culture and handling of the Methanosarcina sp. as a result of the equipment set up under the Contract. We have also made the anaerobic culture facility available to other investigators at the University when it is not in use for the ONR sponsored research.

5. Publications

There is one manuscript submitted and one in preparation that describe the research efforts to date. They are:

Sowers, K.R., and R.P. Gunsalus. 1987. A novel adaption for growth at various saline concentrations by the Archaebacterium Methanosarcina thermophila. J. Bacteriol. (submitted)

Sowers, K.R., and R.P. Gunsalus. 1987. Plasmid DNA from the acetotrophic methanogen Methanocarcina acetivorans C2A. J. Bacteriol. (in preparation).

Sowers, K.R., and R.P. Gunsalus. 1987. A novel phenotype of the methanogenic archaebacterium, Methanosarcina thermophila. Invited talk presented at the 1987 Gordon Conference on Methanogenesis, New Hampton School, New Hampton, NH.

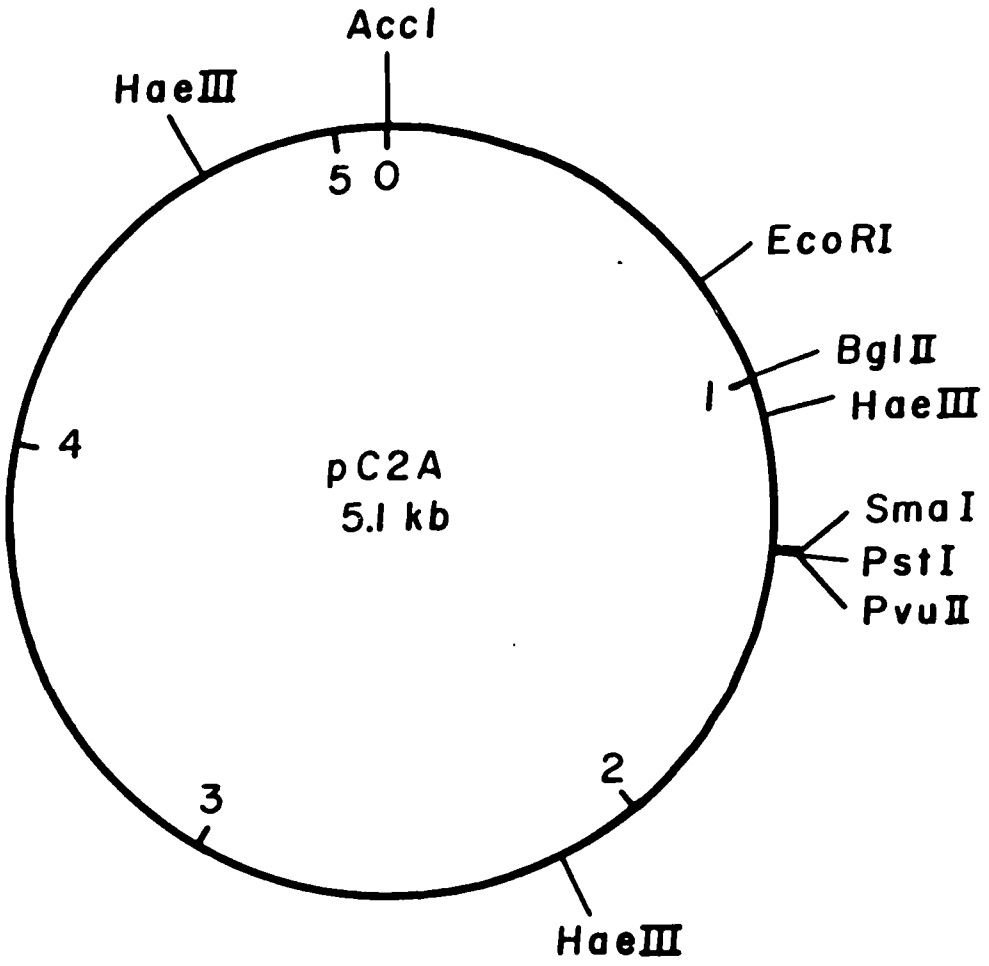

Figure 1. Map of plasmid pC2A from Methanosarcina acitivorans.

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